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13. ABSTRACT (Maximum 200 Words)

Proteins containing a Caspase-Associated Recruitment Domain (CARD) have previously been shown to serve as key regulators of tumor cell survival as well as regulators of other cellular processes, such as cytokine production. Interleukin-1 beta (IL-1B) is a cytokine which has been found to be expressed in breast cancer cells and may be associated with more aggressive and invasive breast tumors. Here we report the cloning and functional characterization of NAC-X or CLAN (CARD, LRR, And NACHT-containing protein). CLAN was found to be expressed in several breast cancer cell lines as well as in monocytes by RT-PCR. Co-immunoprecipitation studies revealed that CLAN associated several other proteins including caspase-1, Nod2, and NAC. When assayed using an IL-1B ELISA, CLAN was found to induce the activation of caspase-1 in response to bacterial infection or LPS, implying a role for this protein in the innate immune response. CLAN also was found to enhance cell death following bacterial infection independently of caspase-1. Through its interactions with other CARD-containing proteins, CLAN may regulate the survival of breast cancer cells and could be utilized as a novel anti-tumor target or diagnostic/prognostic biomarker.

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Apoptosis, CARD proteins, CED-4 homologues, caspase-1, IL-1B

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Introduction

Proteins containing a Caspase-Associated Recruitment Domain (CARD) have previously been shown to serve as key regulators of tumor cell survival as well as regulators of other cellular processes, such as cytokine production. Interleukin-1 beta (IL-1β) is a pro-inflammatory cytokine which has been found to be associated with more aggressive and invasive breast tumors (Jin, Yuan et al. 1997), (Kurtzman, Anderson et al. 1999). Previous work (years 1 and 2) focused on the initial cloning and functional characterization of NAC-X (now named CLAN), a novel protein containing CARD, NACHT and LRR domains. NAC-X was found to regulate caspase-1 and IL-1β activation, and to also affect the functions of other NACHT-containing proteins via heterotypic associations through its NACHT domain. Futhermore, NAC-X was found to elicit an inflammatory response in macrophages following exposure to the bacterial component LPS, suggesting a role for this gene in the innate immune system. In the final year of this fellowship, NAC-X was found to induce an immune response (IL-1β secretion) from macrophages following exposure to bacterial infection. It was also discovered that NAC-X protects cells from bacterial infection and in some cases may cause the host cell to die in response to bacterial invasion.

Body

Specific Aim 1:
Specific Aim 1 of this research project was to determine the expression pattern of NAC-X in normal and malignant mammary tissues as well as in normal human tissues. Tasks 1 and 2 were completed during the first year of the award (screening of NAC-X expression and cloning of full-length NAC-X, respsectively). The goal of Task 3 was to genereate a polyclonal antibody against NAC-X for use in immunoblotting or immunohistochemistry. The first peptide used for immunization failed to produce a viable NAC-X antibody. A second attempt using a different peptide resulted in an antibody which is capable of detecting over-expressed, but not endogenous, NAC-X using western blotting. This antibody is, however, capable of immunoprecipitating CLAN.

Specific Aim 2:
The goal of Specific Aim 2 was to evaluate the associations of NAC-X with other CARD-containing proteins involved in apoptosis. This aim was completed in year 1, leading to the discovery that NAC-X associated with caspase-1, Nod1, Nod2, and NAC. NAC-X was also found to oligomerize with itself through its NACHT (nucleotide-binding) domain. In an effort to expand on this specific aim, the ability of the NACHT domain of NAC-X to mediate binding to other NACHT family proteins was investigated. The NACHT domain of NAC-X was found to hetero-oligomerize with the NACHT domains of Nod2, NAC, and crypoyrin (as well as other proteins within this family, data not shown) but not to several proteins lacking a NACHT domain.

Specific Aim 3:

Specific Aim 3 was to determine the specific step(s) in apoptosis pathways regulated by NAC-X. Work in year one determined that NAC-X does not significantly affect BAX- or fas-mediated cell death but does enhance caspase-1-mediated apoptosis. Another pathway by which cells regulate gene expression and survival is through the activation of the NF-κB transcription complex. Since the Nod proteins have been shown to be inducers of NF-κB, (Inohara, Koseki et al. 1999), (Ogura, Inohara et al. 2001), we next investigated the potential effects of NAC-X on this signaling pathway. It was found that NAC-X was capable of inhibiting Nod1- and Nod2-mediated NF-kB activation in a dose-dependent manner. Since NAC-X and Nod2 are both expressed in monocytes, these proteins may actually play more of a role in the inflammatory process than in apoptosis, as the more recent literature would suggest (Inohara, Ogura et al. 2001).

Specific Aim 4:

The goal of Specific Aim 4 was to analyze the importance of the NAC-X gene for cell survival/cell death *in vivo*. Since recent data has pointed to a role for CLAN in the activation of caspase-1/interleukin-1β, as opposed to its direct involvement in apoptosis, a monocytic cell line over-expressing the full-length NAC-X gene was generated. To accomplish this, a retrovirus was utilized to introduce epitope-tagged NAC-X into THP-1 cells which were subsequently selected for stable expression using G418. To evaluate the effects of NAC-X on the function on monocytes/macrophages, the secretion of endogenous interleukin-1β (IL-1β) was examined by ELISA following treatment with bacterial lipopolysaccharide (LPS), a known inducer of this cytokine in macrophages. It was found that NAC-X enhances the secretion of this pro-inflammatory cytokine. Additionally, infection of THP-1/NAC-X cells with pathogenic bacteria (*Salmonella Enteritidis* and *Salmonella Typhimurium*) resulted in enhanced secretion of IL-1β when compared to THP-1/Neo control cells (figure 1). Conversely, a THP-1 cell line expressing a NAC-X specific RNAi hairpin construct (and subsequently lower levels of endogenous CLAN) was shown to have a IL-1β secretion defect in the same experiments. NAC-X also inhibited the ability of *Salmonella* species to replicate within macrophages, indicating an additional anti-bacterial role for this protein in the innate immune response (figure 2). Finally, NAC-X was found to sensitize macrophages to Salmonella-induced cell death following infections using a higher MOI (multiplicity of infection). To further investigate whether or not caspase-1 was involved in this phenomenon, cells were pre-treated with the caspase-1 inhibitor z-WEHD-fmk or the pan-caspase inhibitor z-VAD-fmk prior to bacterial exposure. Interestingly, cell death mediated by NAC-X seemed to be independent of caspase-1 but dependent on the function of other caspases (figure 3).

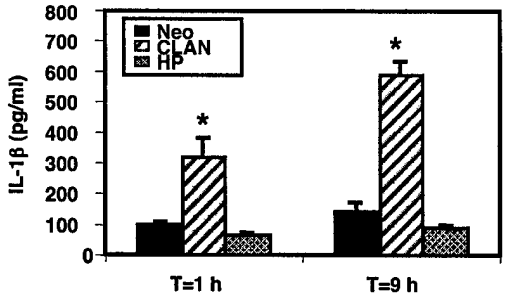

Figure 1. NAC-X (CLAN) N mediates IL-1β secretion from macrophages following bacterial infection. THP-1 cells expressing NAC-X (CLAN), a RNAi hairpin construct specific for NAC-X (HP), or control cells (Neo) were exposed to *Salmonella enteritidis* (at MOI=5) and supernatants collected at t= 1hr and 9hr. Levels of secreted active IL-1β were determined by ELISA.

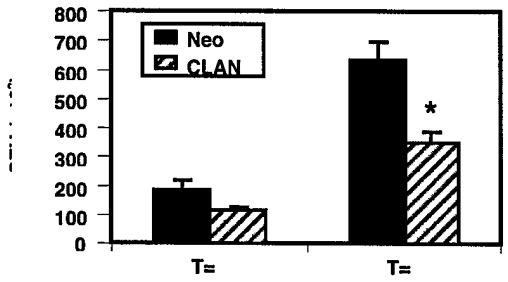

Figure 2. NAC-X inhibits the ability of *Salmonella* to replicate within macrophages. THP-1 cells over-expressing NAC-X (CLAN) or control cells (Neo) were exposed to Salmonella for 1hr, extracellular bacteria was killed using gentamycin, and cells were lysed in 1% triton-X or incubated for an additional 8hr at 37° C. Lysates were spread on LB-agar plates and amounts of intracellular bacteria were determined through CFU numbers.

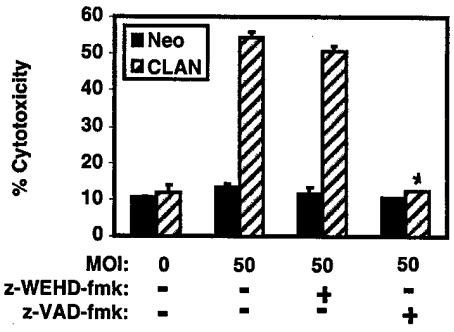

Figure 3. NAC-X sensitizes monocytes to bacteria-induced cell death independently of caspase-1. THP-1 cells were pre-treated with caspase-1 inhibitor z-WEHD-fmk or pan-caspase inhibitor z-VAD-fmk, then were infected with *Salmonella.* Cell death was quantitated through the measurement of LDH release.

Key Research Accomplishments (final year)

*NAC-X was found to mediate IL-1β secretion from macrphages following pathogenic bacteria infection.

*It was determined that NAC-X induces anti-bacterial effects within infected macrophages and thus plays an important role in the innate immune system.

*NAC-X was found to sensitize macrophages to cell death following infection by high levels of bacteria, a process discovered to be independent of caspase-1.

Reportable Outcomes

Damiano JS, Oliveira V, Welsh K, Reed JC. Heterotypic interactions among NACHT domains: implications for regulation of innate immune responses. Biochemical Journal, April 2004 in press.

Jason S. Damiano, Christian Stehlik, Frederick Pio, Adam Godzik, and John C. Reed. Cloning and Characterizaion of CLAN, a novel CED-4 homolog which regulates caspase-1 activity. Dept. Of Defense Breast Cancer Research Program Meeting, 2002.

Conclusions

During the first year of research, NAC-X was successfully cloned and found to be expressed in a number of human tissues, including breast cancer cell lines. Due to its homology with the cell death regulator Apaf-1, NAC-X was initially believed to play a role in determining apoptotic susceptibility in human cells. However, subsequent experiments determined that it was actually a regulator of caspase-1, a caspase not usually involved in apoptosis. This caspase is known to control the cleavage and secretion of pro-IL-1β, a pro-inflammatory cytokine that is key to the innate immune response and possibly associated with a more aggressive breast cancer phenotype. Work done during the initial 2 years lead to the successful generation of a polyclonal antibody against NAC-X. Studies found that NAC-X may affect other similarly structured proteins (of the NACHT family) through interactions mediated by its NACHT domain. Experiments utilizing monocytic cell lines stably expressing NAC-X demonstrated that this protein is capable of mediating cytokine release following stimulation with bacterial components such as LPS. Finally, NAC-X was found to enhance the release of IL-1β following bacterial infection, to inhibit the ability of bacteria to successfully replicate within macrophages, and to sensitize macrophages to death mediated by high amounts of bacteria. Further studies utilizing NAC-X -over-expressing breast cancer cell lines will determine whether or not NAC-X is a mediator of IL-1β secretion in breast tumors. In conclusion, NAC-X is a regulator of the pro-inflammatory cytokine IL-1β, which is known to be an important element of the innate immune response and possibly a survival/growth factor for breast cancer cells.

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